The Histochemical Distribution of Placental Calcium and Alkaline Phosphatase Activity Following Fetoplacental Dissociation in the Albino Rat

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THE HISTOCHEMICAL DISTRIBUTION OF PLACENTAL CALCIUM
AND ALKALINE PHOSPHATASE ACTIVITY FOLLOWING FETOPLACENTAL
DISSOCIATION IN THE ALBINO RAT

by

Eric Sigmond

A Thesis Submitted to the Faculty of the Graduate School
of Loyola University of Chicago in Partial Fulfillment
of the Requirements for the Degree of
Master of Science

February
1976
ACKNOWLEDGEMENT

I wish to express my gratitude to Dr. Leslie A. Emmert for his suggestion of the problem, his patience, encouragement and supervision throughout the course of this thesis. His guidance helped overcome many problems which arose during the course of this study.

I also wish to express thanks to Dr. Charles C.C. O'Morchoe and Dr. Maurice V. L'Heureux for their many valuable suggestions during the writing of this thesis.

Finally, I wish to express my warmest appreciation to my parents for their continued expressions of confidence.
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INTRODUCTION

Calcium transport across the placental barrier has been the subject of numerous studies and reviews (Plumlee 1952, Hallman and Salmi 1953, Feaster 1956, Armstrong et al 1970, Braithwaite et al 1972, Whelan et al 1972, and Hartsook and Hershberger 1973). The important role of calcium in various biological and physiological processes as diverse as muscle contraction and blood coagulation makes knowledge of its transport mechanism of value. When the concentration of calcium in fetal plasma is shown to be greater than that in the maternal plasma (Bogert and Plass 1923, Armstrong et al 1970), an enzymatic active transport hypothesis emerges (Armstrong et al 1970, Shami and Radde 1971, and Miller and Berndt 1973). The general purpose of the present study is to investigate the morphological relationship of alkaline phosphatase to the transport of calcium across the placental barrier.

Calcification of the rat placenta does not normally occur. However, it has been shown that when the umbilical vessels are ligated, the placenta becomes calcified (Emmert 1954). Thus an experimental model has been established in which calcification can be induced and which can be used to study calcium transport in the rat placenta. The experimental hypothesis assumes that sites of calcification in the placenta indicates sites of calcium transfer. An enzyme can then be implicated in the active transport of calcium if it is localized in the same areas as are calcium deposits. Earlier work indicates that
alkaline phosphatase is localized in areas of the placenta similar to those in which calcium deposits form (Wislocki et al 1946). However, correlation between alkaline phosphatase activity and placental calcification has not been previously described. The present study compares the morphological distribution of calcium deposits with the morphological distribution of alkaline phosphatase by selective staining of adjacent sections for alkaline phosphatase activity and calcium deposition.

A knowledge of the transport mechanism may aid in an understanding of the reasons for placental calcification which would, in turn, have clinical implications. If calcification is the result of fetal distress or placental insufficiency, as has been suggested (Fox 1964), an investigation of the placenta at birth could lead to prompt treatment for problems arising from these conditions.
REVIEW OF LITERATURE

Normal Rat Placental Structure

The major contributing investigations of the normal structure of the albino rat placenta were conducted by Duval (1892), Bridgeman (1948), Jollie (1964), and Davies and Glasser (1968). The consensus of their investigations was that grossly, the placenta from 15 to 20 days of gestation is a button-shaped disc, approximately 8 to 10 mm in diameter with a thickness of 2 to 4 mm. Figure 1 illustrates the relationship of the various areas of the placental and uterine structures. Its convex surface borders the junctional zone of the uterine decidua basalis which is a dense layer of fusiform cells that marks the limit of placental invasion of the uterine wall. The placenta is composed of three zones; the spongy zone, the labyrinth and the giant cell ring. The spongy zone is nearest the maternal tissue, lying adjacent to the decidua basalis and extending to the periphery of the placenta. The spongy zone is composed of two types of cells, spongy zone cells and glycogen cells. The spongy zone cells (when stained with hematoxylin and eosin) have darkly staining basophilic nuclei and basophilic cytoplasm. However, their function is unknown. The glycogen cells (when stained with hematoxylin and eosin) have dark basophilic nuclei with clear cytoplasm. These cells may serve a nutritive reserve function by virtue of the glycogen stored in the apparently clear cytoplasm. The glycogen cells occur in islands surrounded by the spongy zone.
cells and degenerate during gestation releasing the cytoplasmic glycogen into the maternal blood coursing to the site of maternal-fetal exchange in the labyrinth.

The labyrinth is adjacent to the fetal side of the spongy zone. Developmentally, the labyrinth arises from a series of trophoblastic projections into the uterine wall which are analogous to the villi in a human placenta. These projections then spread laterally and join, forming a series of interconnecting labyrinthine plates. The spaces between these plates form the maternal blood sinuses while the fetal capillary system penetrates and vascularizes the fetal chorionic connective tissue core of the plates (Fig.2). The maternal blood is thus separated from the fetal blood by the tissue of the labyrinth forming the basis of the adjacent circulations necessary for exchange of nutrients. By electronmicroscopy, Jollie (1964) established that there are three layers of trophoblastic cells between the maternal sinus and the fetal capillaries. However, these are not always resolvable with the light microscope. Thus the metabolic products and nutrients must traverse five layers, the three trophoblastic layers and the capillary endothelium with its basal lamina in order to pass between the maternal blood stream and the fetal circulation.

The giant cell ring girds the placental disc. It is composed of the last of the early trophoblastic invasive cells which allowed for placental expansion into the decidua basalis.

Placental circulation is divided into maternal and fetal portions. The fetal circulation branches from the umbilical vessels which
connect the internal circulation of the fetus with that of the placenta. Upon reaching the placenta, the umbilical artery divides in a radial fashion sending branches to the periphery. These branches, surrounded for a distance by an endodermal lining, penetrate the labyrinth. This lining forms the endodermal sinus of Duval. The branches continue to divide until they form capillaries within the labyrinthine plates. The venous return parallels the arterial circulation in a retrograde manner. The maternal circulation stems from a large maternal artery coursing through the center of the spongy zone and labyrinth. In the fetal side of the labyrinth the blood flows out of the artery and percolates between the labyrinthine plates in the maternal sinuses. A venous sinus collects the blood at the periphery of the placenta and drains to the uterine veins.

** Interruption of Fetal Circulation **

When fetal blood flow stops, there are specific changes that occur within the placenta. Huggett and Pritchard (1945) used hormones to kill rat fetuses in utero. They observed, following the death of the fetuses after the 12th day of gestation that the placenta continued to increase in size and maintained a grossly normal appearance. Emmert (1954) using the technique of Noer and Mossman (1947), ligated the umbilical cord to stop fetal blood flow. Although the placenta appeared grossly normal, he found that the labyrinthine connective tissue was infiltrated by basophilic granular material and that deposits of calcium and iron formed in the thickened labyrinthine syncytiotrophoblast as
well. He also found that fetal capillaries were obliterated and eventually maternal sinuses were often occluded by the thickening labyrinthine syncytiotrophoblast and fibrin deposits. Davies and Glasser (1968) reported parallel results. They found that the placenta continued to grow and differentiate for one to two days after cord ligation and the fetal vessels collapsed and degenerated within four days. They observed the effect of cord ligation on the three layers of trophoblast established by Jollie (1964). Light and electronmicroscopy revealed that the two layers nearest the fetal capillary formed multinucleated masses within two days, while the layer immediately surrounding the maternal sinus was found to persist. These four studies established that although degenerative changes occur in the placenta after cessation of fetal blood flow, the placental cells do continue to maintain normal morphological appearance for one to two days.

**Calcium Distribution**

Calcium distribution in the normal rat placenta was studied by Wislocki et al (1946). They found acid soluble argyrophilic deposits which they interpreted as calcium in the syncytiotrophoblast and in the decidual zones. Emmert (1954) and Schmitz (1966) studied calcification of the rat placenta after ligation of the fetal umbilical cord and found calcium deposits in the labyrinth within 24 hours.

Calcification of the human placenta was studied by Wislocki and Dempsey (1946). They found calcium deposits in the stroma of the chorionic villi immediately beneath the trophoblastic epithelium.
Croley (1973) observed with the electron microscope calcium deposits within the cells of the human placenta. Calcium deposits in the form of vesicles were associated with the rough endoplasmic reticulum as well as the mitochondria of the cells. The mitochondria associated with calcium vesicles were most frequently found near the endothelium of the fetal capillaries. Croley (1973) concluded that the granular deposits represented the location of the calcium as it was being actively transported across the placental barrier within the cells.

The relationship of placental calcification to fetal abnormalities has been the object of other studies (Fox 1964, Tindall and Scott 1965, Wentworth 1965, Tapp 1969, and Avery and Alterman 1971). Tapp (1969) reported a narrow band of calcification immediately beneath the trophoblastic epithelium in the placenta of a still-born, anencephalic child with spinal bifida. He considered calcification to be unusual in the human placenta. Avery and Alterman (1971) countered Tapp's (1969) assertion that placental calcification was unusual in the human placenta because they found deposits in six of thirty placentae studied. In three earlier and more extensive studies, Fox (1964), Tindall and Scott (1965) and Wentworth (1965) described the relationship of placental calcification to a number of fetal and maternal variables. Fox (1964) in a study of 195 placentae found 24.6% to contain easily visible calcium deposits and termed these "calcified." He determined that the percentage of calcified primigravid placentae was significantly higher than the percentage of calcified multigravid placentae. Although the amount of calcification was found to increase as the placenta matured,
excessive calcification was not linked to postmaturity. He also determined that there was no relationship between maternal age and frequency of calcification in placentae. Finally, an increase in placental calcium was found to be correlated with an increase in fetal distress and neonatal asphyxia. This latter observation led Fox (1964) to hypothesize that calcification is a sign of possible complications for the newborn. Wentworth (1965) in a study of 679 placentae found 32.9% of them to contain deposits of calcium which were visible macroscopically in serial sections of 400 micra in thickness. His study agreed with Fox's observation of increased frequency of calcification in primigravid placentae when compared with multigravid placentae and also with the finding that excessive calcification was not a sign of postmaturity. The study disagreed, however, with Fox's finding on maternal age and calcification in that placental calcification, according to Wentworth (1965), decreased with increasing maternal age. Wentworth's (1965) results which showed no correlation between still-births and placental calcification appeared to contradict Fox's (1964) hypothesis that calcification indicates possible complications in the newborn. In an extensive study Tindall and Scott (1965) found 44% of 3025 placentae to contain at least two localized sites of calcification as determined by X-rays of the placentae. Their findings supported: (i) the relationship between gravidity and calcification reported by Fox (1964) and Wentworth (1965); (ii) the relationship between calcification and placental maturity as reported by Fox (1964); and (iii) the inverse relationship between maternal age and calcification as reported by Wentworth (1965). Tindall and Scott (1965) showed there was less calcification in
placentae associated with still-births tending to contradict previous observations (Fox 1964). Because of the normally high incidence of placental calcification and the decrease in calcification in still-birth placentae, Tindall and Scott (1965) concluded that placental calcification was a normal physiological process.

Placental Calcium Transport

Calcium transport across the placenta was at first thought to be passive with the calcium ions moving from a higher concentration in the maternal blood to a lower concentration in the fetal blood. However, this mechanism was disproved by Bogert and Plass (1923) when they found that the concentration of the calcium ions in maternal blood was less than that in fetal blood. Hallman and Salmi (1953) and Armstrong et al (1970) showed similar results in their studies. Armstrong (1970) proposed two explanations for the concentration difference. The first and most obvious was an active calcium transport. The second was that the fetus had homeostatic mechanisms which hold the calcium ion concentration in the fetal blood at a higher level.

The direction of calcium transport across the placenta was studied by Wasserman (1957) and Braithwaite et al (1972). Wasserman (1957) measured discrimination by the rat placenta between radioactive calcium ions and radioactive strontium ions. He found that the rat fetus incorporated ions in a ratio of 2:1 calcium to strontium. However, when the ions were injected into the fetus, there was no difference in the rate at which they were transferred to the mother. One
explanation for these data is that the calcium pump is unidirectional. Much stronger evidence for the orientation of the calcium pump came from a study by Braithwaite et al (1972). The authors injected radioactive calcium into the umbilical vein of a sheep fetus and found no radioactive calcium transferred to the mother. From this evidence, Braithwaite et al (1972) concluded that the transfer of calcium occurs only from the mother to the fetus.

Studies utilizing radioisotopes have also been made to measure the rate of uptake of calcium by the fetus from maternal blood. Feaster (1956) observed rats during the 14th to the 22nd day of gestation. The amount of calcium transferred across the placenta increased with increasing fetal age. Comar (1956) found that in the rat during late gestation an equivalent of 100% of the maternal blood calcium was transferred to the fetus in one hour. The percentage of maternal blood calcium transferred to the human fetus in the same amount of time is an equivalent of 7% of the maternal blood calcium. The figure for the bovine fetus is 50%. Twardock (1967) determined that the guinea pig fetus removed the equivalent of 400% of the maternal blood calcium per hour during late gestation. Whelan et al (1972) found that in deer, the amount of radioactive calcium transferred across the placenta increased with increasing fetal age. The same findings were reported by Braithwaite et al (1972) for calcium transfer in sheep.

Due to the rapid rate of transfer of calcium, the source of calcium in the mother for fetal use was of interest. Studies by Newman (1943) and Mull and Bill (1934) showed that a depression in maternal blood levels of calcium in humans was most severe during the sixth or
seventh week before delivery. This was interpreted as evidence that at the time significant amounts of maternal plasma calcium were being transferred to the fetus. Feaster (1956) concluded that in the rat, part of the calcium transported across the placenta was derived from the maternal skeleton. Wasserman et al (1957) indicated that for the rat and rabbit 92% of the calcium came from the maternal diet and 8% came from the maternal skeleton. Whelan et al (1972) suggested that in deer there was also a calcium pool with a "lower specific activity" than that of injected radioactive plasma calcium which served as the major source of fetal calcium. From these divergent sources, it is apparent that while the maternal blood supplies some calcium, there must be another calcium pool which is the major source of fetal calcium. The maternal skeleton most probably serves as this source.

Placental Alkaline Phosphatase

The distribution of alkaline phosphatase in the placenta has been studied on the gross, microscopic, and ultrastructural levels. On the gross level all studies have shown alkaline phosphatase activity in the region where transfer of nutrients takes place. Hard (1946) found alkaline phosphatase activity in the labyrinth of the guinea pig placenta. Wislocki et al (1946) and Curzen (1964) found alkaline phosphatase activity in the syncytium of the human placenta. Padykula (1958) observed activity in the cytoplasm of the trophoblast in the rat placenta. At the microscopic level, Pritchard (1947) and Padykula (1958) found that the alkaline phosphatase activity lined the maternal sinuses
in the rat placenta. Wielenga and Willighagen (1962) reported that alkaline phosphatase activity was most intense on the maternal side of the villi of the human placenta and weakest on the side towards the basal lamina. The intracellular distribution of alkaline phosphatase was studied by Zilfstra et al (1970) and Hulstaert et al (1973). Zilfstra et al (1970) crushed human placental cells and centrifuged them. They tested the different fractions for alkaline phosphatase activity. They found the alkaline phosphatase activity to be localized in the microsomal fraction. This indicated that the enzyme was located either in the endoplasmic reticulum or in the cell membrane. Hulstaert et al (1973) investigated the intracellular distribution of alkaline phosphatase in the human placenta with the electron microscope. They found alkaline phosphatase activity localized: (i) along the plasma membrane lining the microvilli; (ii) in vesicles of unknown origin within the syncytiotrophoblast; (iii) and along part of the basal lamina.

A biochemical study of the activity of alkaline phosphatase before and after umbilical cord ligation in the rat was performed by Aoba et al (1972). Placentae were harvested 4, 8, and 12 hours after umbilical cord ligation on the 18th day of gestation. The placental material was homogenized and incubated for 60 minutes with phenolphthalein diphosphate. Photometric readings were taken on the supernate after the homogenate was centrifuged. For each of the three groups of placentae, the experimental readings were within the standard deviation of the control readings. For the placentae harvested 4, 8, and 12 hours after umbilical cord ligation, the activity of the experimental placental alkaline phosphatase was 89.4%, and 95.5% of the control activity.
respectively. This experiment showed that the alkaline phosphatase activity was not significantly affected by umbilical cord ligation during the first 12 hours after the ligation occurred.

Alkaline phosphatase, localized in the placenta, has been implicated in the transport mechanism of the placenta (Hard 1946). Alkaline phosphatase was also observed in the kidney and intestine where reabsorption and absorption, respectively, take place. These circumstances immediately suggest that it may serve a similar function in the placenta (Hard 1946). Hard (1946) also noted that alkaline phosphatase was located only in the labyrinth where the exchange of nutrients between mother and fetus occurred. He then drew the analogy between the placenta, kidney and intestine by noting that in each case, alkaline phosphatase was confined to the membrane structure. Ahmed (1959) also noted that the activity of the placenta was similar to that of the kidney and intestine. Since alkaline phosphatase was found in these areas, Ahmed (1959) hypothesized a similar function for the enzyme in the three organs. Khattab and Forfar (1971) while studying placental insufficiency, found lower than normal levels of calcium and glucose in the umbilical cord blood at birth and in the infant's blood four days after birth. Because of the consistent correlation between the blood levels of calcium and glucose in these infants, the authors suggested that the placental insufficiency was due to the failure of a coupled transport mechanism for calcium and glucose. Wislocki et al (1946) drew an analogy between bone metabolism and calcium and alkaline phosphatase in the placenta. They suggested that since calcium was found near alkaline phosphatase and glycogen deposits, the relationship
of the three might be analogous to bone metabolism. According to this theory, glycogen would serve as the source of glycopshosphoric acid esters. This in turn would serve as the substrate for the alkaline phosphatase producing the phosphate groups used in calcification. However, this hypothesis would not assign a function of calcium transport to alkaline phosphatase unless it could be shown that calcium was transported in the form of calcium phosphate.

Two investigators, (Shami and Radde 1971, Miller and Berndt 1973) isolated ATPases from placental tissue that were activated by calcium and that had an optimum pH in the range of 8.0 - 8.5. Shami and Radde (1971) isolated a calcium activated ATPase from the membranes of the guinea pig placenta. Its optimum pH was 8.2 - 8.5. Miller and Berndt (1973) isolated a calcium activated ATPase from the human placenta with an optimum pH of 8.0. Both suggested that the enzymes were responsible for the active transport of calcium. These biochemical studies render a functional significance to previous morphological investigations (Wislocki et al 1946 and Wislocki and Dempsey 1946) which found calcium and alkaline phosphatase activity in the same localities in the human and rat placentae.
OBJECTIVES

The aims of the present study were:

1. to determine the effects of umbilical cord ligation on placental morphology;
2. to investigate and compare histochemically the calcium distribution in normal and cord ligated placentae and determine the effect of the duration of umbilical cord ligation on calcification of the placentae;
3. to compare the sensitivities of the GBHA calcium staining method of Kashiwa and House (1964) and the alizarin red calcium staining method of Dahl (1952) and determine if the more sensitive GBHA method revealed more calcium than did the alizarin red method;
4. to investigate the intensity and distribution of alkaline phosphatase activity as measured by the staining method of Burstone (1958);
5. to compare the distribution of alkaline phosphatase activity with the distribution of calcium within the placenta.
MATERIALS AND METHODS

The rats used in the present experiment were of the Sprague-Dawley strain and were obtained from Locke-Erikson Laboratories, Maywood, Illinois. Of the total of 22 female rats used in the experiment, 9 were bred at the Loyola Medical Center. Females were placed with males overnight and vaginal smears were taken in the morning. The presence of spermatoza in the smear was used as an indication for the onset of pregnancy. The remaining 13 female rats were purchased dated sperm positive.

Results were obtained from the study of more than 156 placentae, more than 50 of which served as controls with the rest being approximately evenly divided among the experimental groups (Tables 1, 2 and 3). The alkaline phosphatase stain, the GBHA calcium stain and the alizarin red calcium stain were applied only to the paraffin embedded freeze-dried sections while the specimens fixed with buffered formalin were used for morphological studies and stained only with hematoxylin and eosin.

A standard operation was performed on all rats. Each rat was anesthetized with ether and its abdomen shaved. Ninety-five percent ethanol was poured over the surgical area to aid in antisepsis. An incision was made just lateral to the midline of the abdomen. On each side of the incision folded bibulous paper soaked in a 0.9% saline was placed in preparation for the uterine horns. Each horn of the uterus was exposed individually and placed on the bibulous paper. A dissecting
microscope light was used to transilluminate the conceptus. A small curved needle and thin cotton thread were passed through the uterine wall, around the umbilical cord, and back through the wall. The thread was drawn tight and secured, ligating the umbilical cord. Umbilical cord ligation was performed on approximately two thirds of the conceptuses in each animal. The remaining one third served as unoperated controls. The uterine horns were then returned to the abdominal cavity and the incision closed in layers.

The animals underwent surgery on either the 15th, 16th, or 19th days of gestation. Placentae were harvested 24 and 48 hours after operations on the 15th and 16th days of gestation and 24 hours after operations on the 19th day of gestation. Thus there were five groups of animals: (i) those operated on the 15th day and harvested 24 hours later (15/24); (ii) those operated on the 15th day and harvested 48 hours later (15/48); (iii) those operated on the 16th day and harvested 24 hours later (16/24); (iv) those operated on the 16th day and harvested 48 hours later (16/48); and (v) those operated on the 19th day and harvested 24 hours later (19/24).

To harvest the placentae, the rat was anesthetized with ether and the original incision of the operation was extended into the thoracic cavity causing a pneumothorax. The uterus was removed and each horn was slit longitudinally on the side opposite to the implanted placentae. The placentae were removed by dissection with the uterine wall still attached and then cut in half. One half was placed in isopentane cooled in liquid nitrogen and the other was placed in
phosphate buffered formalin (Humason 1962). The 16/24 group was fixed in a gluteraldehyde solution but this unfortunately yielded poor results, so the remaining specimens were fixed in buffered formalin. The frozen tissues were stored at -69°C until they could be dehydrated in an Edwards EPD 3 tissue drier. In the drier the placentae were kept under a pressure of 0.1 - 0.01 torr at a temperature near -70°C at least overnight to dry and then infiltrated with paraffin without breaking the low pressures. The tissues in the buffered formalin were fixed for 24 hours, washed in tap water, dehydrated in increasing steps of graded ethanol, infiltrated with paraffin in the vacuum oven and embedded in paraffin.

The tissues were sectioned on an American Optical 820 microtome such that the sections were approximately through the center of the placenta and parallel to the maternal-fetal axis. In each case three sequential sections were placed on three slides forming a set. Approximately 10 sections were then discarded and three additional sequential sections were placed on three slides. For the freeze-dried material it was necessary to use a small pool of water on a slide to flatten the tissue section. The water then had to be removed immediately after the section was placed on the slide in order to avoid the absorption of excess water with consequent damage to the tissue. Initially the tissues were sectioned at 8 micra but this was reduced to 6 micra in order to discern placental structures more clearly.

Two sets of slides from each placenta were stained. In the first set, the first slide was stained using the hematoxylin and eosin method of Gill et al (1974), the second was stained by the alizarin red
method of Dahl (1952) for calcium and the third was stained by the AS-MX naphthol phosphate method of Burstone (1958) for alkaline phosphatase activity. In the second set, the first slide was stained with the GBHA method of Kashiwa and House (1964) for calcium, the second was stained with the alkaline phosphatase method of Burstone (1958) and the third was stained with the Dahl (1952) method for calcium.

The alizarin red calcium stain was obtained by adding 0.5 gm of alizarine red S to 45 ml of distilled water. Five milliliters of a 1:100 dilution of 28% ammonium hydroxide solution was then slowly dripped into the alizarin red solution as it was stirred. Two xylene baths were used to clear the sections and decreasing concentrations of ethanol were used to hydrate the sections. The tissues were stained for at least 2 minutes in the alizarin red mixture. Staining periods exceeding 2 minutes resulted in no increase in the intensity of the staining. The slides were then rinsed by a gentle stream of distilled water and placed directly into 95% ethanol to avoid leaching which occurred in lower concentrations of ethanol. After the sections were cleared in two baths of xylene, they were mounted with HSR mounting medium.

The GBHA calcium stain required the preparation of four stock solutions: (i) 0.4 gm% glyoxal bis (2-hydroxy-anil) ethanol solution; (ii) a 5 gm% NaOH deionized water solution; (iii) a saturated solution of KCN and Na₂CO₃ in 90% ethanol; (iv) and a 0.1 gm% solution of fast green and methylene blue in 95% ethanol. The slides were placed on a flat surface and the tissues still embedded in paraffin were flooded for 15 minutes with a staining solution consisting of 0.3 ml of the
NaOH solution and 2 ml of the alcoholic GBHA solution. The tissues were rinsed in 70% ethanol for 2-3 minutes and dipped in 3 baths of 95% ethanol before being immersed in the alcoholic Na$_2$CO$_3$-KCN solution for 15 minutes. GBHA will complex with Cu, UO$_2$, Ni, Co, Mn, Zn, and Cd ions as well as Ca. However, an alcoholic solution of Na$_2$CO$_3$ and KCN will decolorize GBHA complexes with all these ions except those with calcium. Therefore, the treatment with the bicarbonate-potassium cyanide solution was necessary to insure that only the calcium deposits would be stained. The tissues were rinsed in two changes of 95% ethanol and counterstained for 3 minutes in the alcoholic fast green-methylene blue solution. After three rinses in 95% ethanol and one rinse in absolute ethanol, the tissues were cleared in a 50/50 mixture of ethanol and xylene and two rinses of pure xylene. Mineral oil was used as the coverslip mounting medium because Preservaslide and Clearmount were not available. HSR or Permount leached the stain from the calcium within 12 hours of mounting. To stabilize the coverslip a ring of HSR was added to the edges of the coverslip without detrimental effects upon the stain.

To prepare the AS-MX naphthol phosphatase method (Burstone 1958) for staining alkaline phosphatase activity, 5 mgm of naphthol AS-MX phosphate was dissolved in 0.25 ml of dimethyl formamide and added to 25 ml of distilled water. To this solution was added 25 ml of 0.2 M tris buffer of pH 8.7. Ten milligrams of fast blue RR was added to the 50 ml solution and stirred. The resulting solution was then filtered into the staining dish in order to remove the undissolved particles of fast blue RR. While the staining solution was prepared,
the tissues were hydrated in a sequence of ethanol dilutions identical to those used with the alizarin red. This synchrony was necessary because the final AS-MX solution was unstable, such that the intensity of staining decreased appreciably within about 15 minutes. The tissues were stained for 15 minutes and then rinsed in a distilled water bath. Coverslips were mounted with a water-soluble glycerol-gelatin mixture. Dehydration was avoided because of its blanching effect.

To determine the effect of variation in thickness of the tissue section on the intensity of the various stains, three sets of thirty serial sections were cut, the first set at 6 micra, the second at 7 micra and the third at 8 micra. The first ten slides of each set were stained with the alkaline phosphatase method (Burstone 1958), the second ten slides were stained with alizarin red (Dahl 1952), and the third ten slides were stained with GBHA (Kashiwa and House 1964). The slides were then compared to observe differences in staining intensity related to section thickness. This procedure was also used to observe the consistency of the staining technique by comparing adjacent sections to one another.

The slides were evaluated subjectively on the basis of the amount of stain within the labyrinth of the placenta. Slides were laid on a white sheet of paper and placed into 5 grades, grade 1 having the least amount of stain and grade 5 having the most. The ratings were performed on a double blind basis using two observers.

The morphological correlation between calcium deposits and alkaline phosphatase activity was determined by first viewing the section stained for calcium. The extent of the fields of calcification
was determined and morphological landmarks were noted. The section stained for alkaline phosphatase activity was then viewed using the morphological landmarks for orientation. An estimate was then made of the percentage of the fields of calcification which were in the same localities as the alkaline phosphatase activity. The percentage of correlation was then divided into four groups: 0–25%, 25–50%, 50–75%, and 75–100%.
RESULTS

Morphology of the Control and Cord-Ligated Placentae

Since the labyrinth is the location of nutrient-waste transfer in the placenta, it was the major focus of the present study. The morphology of the control labyrinth is shown in Figures 2 and 3a-7a. The labyrinthine plates formed a network which contained the fetal capillaries and also formed the walls of the maternal sinuses. The three trophoblastic layers described by Jollie (1964) were not seen. The maternal sinus channels were of approximately consistent diameter and radiated from the central maternal artery to join with the venous sinus at the periphery. The maternal sinuses were often cut in a lengthwise fashion, revealing their continuity through the labyrinth.

The pathological changes in the labyrinth associated with umbilical cord ligation are illustrated in Figures 3b-7b. In each age group, the labyrinthine plates were swollen and many fetal capillaries collapsed. This was the only change exhibited by the 15/24 experimental group. The 15/48, 16/24 and 16/48 experimental groups showed more extensive pathological changes. Each group showed areas of basophilic granular material in the stroma of the swollen labyrinthine plates (Figure 6b). Instead of the constant diameter shown by the maternal sinuses in the control specimens, the maternal sinuses in the experimental specimens were enlarged. The maternal sinuses within the experimental placentae were also not cut in a lengthwise fashion as often as
in the controls, indicating a disruption of their normal paths due to the swelling of the labyrinthine plates. The 19/24 experimental group also showed the deposits of basophilic granular material, the labyrinthine plate swelling, and the maternal sinus disruption (Figure 7b). The most characteristic trait of this group, however, was the degeneration of the labyrinthine trophoblastic cell nuclei.

The freeze-dried sections stained with hematoxylin and eosin were paler than the buffered formalin fixed tissues and covered a larger area than did the buffered formalin sections because of water absorption when the tissues were flattened on the slide. The freeze-dried tissue also appeared vacuolated because of ice crystal formation during the freezing procedure. The maternal sinuses were clearly distinguishable although their edges were rougher than in the formalin fixed tissues. It was sometimes difficult to distinguish the fetal capillaries within the labyrinthine walls on the control sections. Because of the vacuolation and the resulting distortion of the freeze-dried sections, the buffered formalin sections were chosen for the morphological studies.

Method Controls

The three sets of adjacent sections cut at 6, 7 and 8 micra and stained with GBHA showed no variation in the distribution of calcium in the labyrinth and no variation in the intensity of the stain when the 6 and 8 micra sections were compared (Table 6). The three sets of adjacent slides stained with alizarin red yielded the same results.
The analogous set of sections stained for alkaline phosphatase activity showed a greater variability in distribution and intensity (Table 6). The 6 and 8 micra groups ranged in intensity from grade 3 to grade 5 and the 7 micra group ranged in intensity from grade 4 to grade 5. Of the sections in the 6 micra group, 82% stained with grade 4 intensity whereas the 7 and 8 micra groups were evenly divided between the grade 4 and grade 5 intensities. The distribution, while remaining constant for the majority of the adjacent sections, did show some variation.

**GBHA Calcium Stain**

Sections stained with GBHA were graded on the following basis: grade 1 (Figure 8) showing no calcium; grade 2 showing calcification only within the walls of the major fetal blood vessels; grades 3, 4 and 5 showing light, moderate and heavy calcification in the labyrinth respectively (Figures 9, 10 and 11). The vast majority of control specimens fell into the grades 1 and 2. In these grades, the labyrinth was stained only with the fast green counterstain (Figure 8). When the initial grading of the GBHA stained tissues occurred, calcification in the major fetal blood vessels was considered to be of some significance. However, when the distribution of the control and experimental placentae of each dated group among the calcification grades was determined, it was found that many of the tissues with calcification in the major fetal blood vessels were derived from control placentae (Table 1 and Graphs 1-5). Therefore, it was concluded that ligation of the umbilical cord
was not responsible for these calcium deposits. Calcification of the labyrinth first appeared in grade 3 placentae in the form of light deposits around maternal sinuses (Figure 9). Calcification intensified in grade 4 and distinctly lined the maternal sinuses (Figure 10). In grade 5 the calcification intensified further and spread into the connective tissue of the labyrinthine plates (Figure 11).

It can be seen from Table 1 and Graphs 1-5 that as gestation increased, so the amount of calcification found in the cord-ligated placentae increased. No labyrinthine calcification was found in the 15/24 experimental group whereas 74% of the 16/24 experimental group showed labyrinthine calcification to some degree. A comparison of the 15/48 experimental group with the 16/48 experimental group showed a similar trend. Thirty-nine percent of the 15/48 group showed labyrinthine calcification of grade 3 and the 16/48 group showed 40% in grade 3 and 33% in grade 4. The 19/24 group showed the highest percentage of tissue sections with calcification and the most intense calcification overall with 57% in grade 5, 19% in grade 4 and 6% in grade 3, making a total of 82% of the sections showing labyrinthine calcification (Table 1 and Graph 5).

When the duration of umbilical cord ligation was compared with the extent of calcification of the placentae, results were somewhat ambiguous (Graphs 1 and 2, Table 1). There was an obvious difference between the 15/24 group and the 15/48 group with 0% of the 15/24 group showing labyrinthine calcification and 39% of the 15/48 group showing grade 3 calcification. However, when comparing the 16/24 and the 16/48
groups, the contrasts were not as sharp. The 16/24 group had 63% of the experimental placentae in grade 3 and 11% in grade 5 whereas the 16/48 group had 40% in grade 3 and 33% in grade 4 (Graphs 3 and 4, Table 1). Although the 16/48 group had a higher percentage of placentae in the more intense calcification grades than did the 16/24 group, the total percentage of sections showing labyrinthine calcification was essentially the same in both groups (74% in the 16/24 group and 73% in the 16/48 group). However, taking into account the high percentage of the 16/48 group placentae with grade 4 calcification, it would seem that the amount of calcification did increase somewhat with increased periods of umbilical cord ligation.

Alizarin Red Calcium Stain

Sections stained with alizarin red were graded in the following manner: grade 1 (Figure 12) showed no labyrinthine calcification; grades 2, 3, 4 and 5 showed light, moderate, moderately heavy, and heavy labyrinthine calcification respectively (Figures 13, 14, 15 and 16). The vast majority of control specimens fell into the grade 1 category showing nothing but the light background stain of alizarin red in the labyrinth (Table 2, Figure 12). The experimental placentae of both the 15/24 and the 15/48 groups showed no calcium deposits using the alizarin red stain. However, all older groups showed labyrinthine calcification in some experimental placentae (Table 2). Of the 16/24 experimental group, 25% showed grade 2 calcification and 6% showed grade 5 calcification. Of the 16/48 experimental group, 29% showed
grade 4 calcification. Of the 19/24 experimental group, 13% showed grade 3 calcification, 15% showed grade 4 calcification, and 55% showed grade 5 calcification. These results showed an increase in calcification corresponding to an increase in gestational age (Table 2). Calcification was also shown to increase somewhat with the increasing duration of umbilical cord ligation.

Comparison of the Alizarin Red and GBHA Calcium Stains

A comparison of the alizarin red and GBHA calcium stains demonstrated that the GBHA revealed more labyrinthine calcification overall than did the alizarin red stain. Although both stains appeared to be comparable in the youngest and oldest experimental groups (15/24 and 19/24) and in all the control groups, the GBHA stain revealed more sites of calcification in the remaining experimental groups (15/48, 16/24, and 16/48) (Table 3 and Graphs 11-14). Both the alizarin red and GBHA showed an absence of labyrinthine calcification in the 15/24 experimental placentae and in the vast majority of control placentae. Essentially the same percentages of experimental placentae showed labyrinthine calcification in the 19/24 group (97% for GBHA and 95% for alizarin red). However, in the 15/48 experimental group GBHA showed labyrinthine calcification in 39% whereas alizarin red showed 0%. In the 16/24 experimental group GBHA showed labyrinthine calcification in 88% of the placentae whereas alizarin red revealed calcium in only 31%. In the 16/48 experimental group GBHA showed labyrinthine calcification in 73% of the placentae whereas alizarin red showed it in only...
Because of the greater sensitivity of GBHA in revealing labyrinthine calcification, it was used in the comparison of the morphological distributions of calcium and alkaline phosphatase.

Distribution of Alkaline Phosphatase

The sections stained for alkaline phosphatase were graded in the following manner: grades 1, 2, 3, 4 and 5 showed weak, moderately weak, moderate, moderately strong, and strong activity respectively (Figures 17-21). Grade 1 activity was not only weak, but also scattered in random patches throughout the labyrinth. In grades 2 through 5 the patches became larger and more intensely staining until in grade 5, the alkaline phosphatase activity covered the labyrinth with the greatest intensity. Activity lined the maternal sinuses and only spread into the labyrinthine plate when it was most intense.

There was no clear cut difference between the alkaline phosphatase activity in the control and experimental placentae (Graphs 6-10). The variation between the most intensely staining experimental and control placentae in each dated group was no greater than the 2-grade variation exhibited by the alkaline phosphatase method control placentae. All dated groups except for the 15/48 group showed no more than a 1-grade difference between the most intensely staining experimental and control placentae. The 15/48 group showed a 2-grade difference between the most intensely staining experimental and control placentae. There was, however, a general increase in the intensity of alkaline phosphatase staining with increased gestational age as
indicated by the general decreases in the percentage of placentae in grades 1 and 2 and a general increase in the percentage of placentae in grades 4 and 5 (Table 4, Graphs 6-10). The only placentae showing grade 5 activity belonged to the 19/24 group, the most mature group of the experiment.

Comparison of Calcium Distribution and the Distribution of Alkaline Phosphatase Activity

The amount of morphological correlation between calcium deposits and alkaline phosphatase activity was found to vary when adjacent sections of tissue were compared. Morphological correlation was defined as the percentage of calcium deposits showing corresponding alkaline phosphatase activity in the same areas of the placenta. In the comparison of sections, the amount of morphological correlation was divided into 4 categories, 0-25%, 25-50%, 50-75% and 75-100%. Graphs 15-18 and Table 5 show the distribution of experimental placentae from each dated group among the four ranges of morphological correlation. Since there was no calcification found in the 15/24 experimental placentae, they were not included in the analysis. Of the 15/48 experimental placentae, 100% showed less than 50% correlation between calcium deposits and areas of alkaline phosphatase activity. The 16/24 and the 16/48 experimental placentae also correlated poorly in that 54% and 58% respectively showed less than 50% correlation between calcium deposits and areas of alkaline phosphatase activity. In the 19/24 group there was a dramatic change. Of the experimental placentae,
82% showed greater than 75% morphological correlation between the calcium deposits and the areas of alkaline phosphatase activity.

Graphs 19-21 and Table 5 show the distribution of the total number of placentae from each of the calcification grades 3 through 5 among the four ranges of morphological correlation. Of the placentae with grade 3 calcification, 74% showed less than 50% correlation. The correlation became better in grade 4 with 56% of the placentae showing more than 50% correlation. In grade 5 the highest correlation existed. One hundred percent of the placentae showed greater than 75% morphological correlation between calcium deposits and alkaline phosphatase activity. The comparison of the amount of morphological correlation between calcium deposits and areas of alkaline phosphatase activity with the dated groups and the calcification grades showed that the amount of correlation increases with increasing gestational age and increasing intensity of calcification.

Comparison of the Intensities of the GBHA Calcium Stain and the Alkaline Phosphatase Stain

Although both the alkaline phosphatase activity and placental calcification showed an increasing trend as gestation progressed, the correlation between the intensity of the stains was not exact (Tables 1 and 4). The 15/24 experimental placentae showed no labyrinthine calcification and correspondingly most showed weak or moderately weak alkaline phosphatase activity. However, 7% of the placentae showed grade 4 alkaline phosphatase activity without highly calcified
counterparts to reflect this increased activity. Of the 15/48 experimental placentae, 39% showed grade 3 calcification. The alkaline phosphatase activity showed a corresponding increase with 13% of the placentae in each of the activity grades 3 and 4. Of the 16/24 experimental placentae, 63% showed grade 3 calcification and 11% showed grade 5 calcification. The alkaline phosphatase activity was not greater than grade 3, although 44% of the placentae were in this category. Again, an increase in the alkaline phosphatase activity followed the increased calcification, but there was no increase to match the 11% of the placentae showing grade 5 calcification. Of the 16/48 experimental placentae, 33% showed grade 4 calcification and 40% showed grade 3 calcification. There was a concomitant rise in the alkaline phosphatase activity with 33% showing grade 4 activity, although only 7% showed grade 3 activity. The correlation between the grade 4 alkaline phosphatase activity and the grade 4 calcium staining intensity was good (33% and 33%), although the grade 3 alkaline phosphatase activity did not correlate well with the grade 3 calcification (7% as opposed to 40%, respectively). The highest correlation between the two staining intensities was exhibited by the 19/24 group. Grades 3, 4 and 5 of alkaline phosphatase activity composed 8%, 11% and 54% respectively of the total number of placentae and grades 3, 4 and 5 of calcium staining intensity composed 6%, 19% and 57% respectively of the total number of placentae. Because of some variability in the intensity of alkaline phosphatase staining, the rise in alkaline phosphatase activity could not be related to the increase in calcification. However, these results show that both the calcium and alkaline phosphatase staining activities
increased during the course of gestation.
DISCUSSION

Calcium

Calcium distribution in the placenta as found in the present study was consistent with the findings of earlier studies (Emmert 1954 and Schmitz 1966). The calcification began around the maternal sinuses in the labyrinth and increased in intensity as gestation progressed. Schmitz (1966) found a similar increase in intensity of calcium staining in the cord ligated placenta in his study on the effects of hypervitaminosis D on calcification in the rat placenta. Emmert (1954) stated that in a fetalectomized placenta, the deposits of calcium occurring in the placenta after the fetalectomy grew more intense the nearer to term the operation was performed. Both Emmert (1954) and Schmitz (1966) found calcification in the labyrinthine stroma adjacent to the maternal sinuses.

Analogous results have been obtained in studies on the human placenta (Wislocki and Dempsey 1946, Fox 1964, and Tindall and Scott 1965). Tindall and Scott (1965) and Fox (1964) found that the amount of calcification in the human placenta increased in direct proportion to advancing placental maturity. Wislocki and Dempsey (1946) found calcium deposits in the stroma of the chorionic villi just beneath the trophoblastic epithelium. Since the villi in the human placenta are analogous to the labyrinthine plates in the rat placenta, these findings are analogous to those of the present study.
Alkaline Phosphatase

The alkaline phosphatase distribution found in the present study, like the calcium distribution, was in agreement with the findings of earlier studies. Alkaline phosphatase was found to line the maternal sinuses of the placental labyrinth when activity was present. The distribution throughout the labyrinth was found to be patchy during early gestation and then to become more uniform and more intense toward the end of gestation. Wislocki et al (1946) found that alkaline phosphatase activity occurred plentifully in the syncytium of the placental labyrinth and that it increased steadily until term. Although the alkaline phosphatase activity found in the control and experimental placentae could not always be termed "plentiful," there always was activity in the trophoblast and this activity did increase as gestation progressed. Padykula (1958) found the alkaline phosphatase activity to line the maternal sinuses although no mention was made of the distribution throughout the labyrinth. The present study agrees with the findings of Schmitz (1966) with respect to the relation between increasing intensity of the alkaline phosphatase staining and increasing gestational age and with respect to the distribution of the alkaline phosphatase in the trophoblast and labyrinth. An apparent conflict was encountered with the results of Pritchard (1947). In Pritchard's (1947) study it was found that: "At the 16th day every maternal blood channel of the rat placenta was clearly defined by lines of intense (alkaline phosphatase) staining." This difference was due to a difference in techniques used in each experiment. Pritchard (1947) cut the sections
used in his study at 8 micra and he stained for alkaline phosphatase using a cobalt sulfide–metal substitution method which gave much more opaque deposits than did the azo dye method used in the present study. The greater intensity of the staining obtained by Pritchard (1947) can therefore be explained by his method of staining. The cobalt sulfide method of staining was not feasible for the present study because of the interference of the calcium deposits in the experimental placentae with the staining process. The cobalt ion, if used, would replace the calcium ion in the calcium deposits as well as mark the areas of alkaline phosphatase activity thus giving false positive results. The greater distribution of alkaline phosphatase activity can be explained by the greater thickness of the sections used in Pritchard's (1947) study. During the course of the present study, some sections were cut at 8 micra and stained for alkaline phosphatase activity. The distribution obtained from these slides was similar to that described by Pritchard (1947). One must assume, therefore, that the extra thickness was responsible for the greater distribution of the alkaline phosphatase activity in Pritchard's (1947) results.

Wislocki and Dempsey (1946) and Curzen (1964) in their studies of the human placenta found that the alkaline phosphatase activity began to appear in the human placenta during the first months of pregnancy and increased steadily until term. The activity was located in the stroma of the chorionic villi. Alkaline phosphatase activity increased with increasing gestational age in both the rat and human placentae and was located in analogous structures in both organs, thus
establishing a parallel between the two types of placentae.

The similarity of alkaline phosphatase staining intensities in the experimental and control placentae are consistent with the results of biochemical determinations obtained by Aoba et al (1972). They found that when the alkaline phosphatase activity of placentae whose umbilical cord had been ligated for 4, 8 and 12 hours was compared with the alkaline phosphatase activity of the control placentae, the values of the experimental activity were within the standard deviation of the control activity. Although not as exact as the biochemical methods of Aoba et al (1972), the results of this experiment showed no apparent difference between the alkaline phosphatase activity of the experimental and control placentae.

Active Transport

Because of the presence of alkaline phosphatase in the rat placenta, analogies have been drawn between the function of alkaline phosphatase in the placenta and the alkaline phosphatase in the kidney and intestine (Hard 1946 and Ahmed 1959). Since the three organs participate in active transport, it was hypothesized that the enzyme serves this function in all three structures. Biochemical isolation from the guinea pig placentae (Shami and Radde 1971) and from the human placenta (Miller and Berndt 1973) of ATPases which were activated by calcium ions and had an optimum pH of 8.2 - 8.5 for the guinea pig and 8.0 for the human was strong evidence for the transport role of alkaline phosphatase. Although the early implications were more speculative, the
isolation of the calcium activated ATPases was strong evidence in favor of alkaline phosphatase being the calcium pump. In a general sense the findings of the present study could be construed to support this notion. Both calcium and alkaline phosphatase were found primarily within the labyrinth and both lined the maternal sinuses of the placental labyrinth. Both were found to increase in intensity of staining as gestation progressed. Without a direct side by side comparison of the distribution of the two, it could be stated that the morphological distribution of both was the same. However, side by side comparison of adjacent sections, one stained for alkaline phosphatase and one stained for calcium showed no consistent correlation between the two. This conclusion was derived mostly from earlier stages of calcification and alkaline phosphatase activity. The random distribution of the alkaline phosphatase activity in the young placentae had the least possibility of coincidental morphological correlation with patches of calcification and therefore gave the best test of morphological correlation. The older placentae whose labyrinths were covered with calcium deposits and alkaline phosphatase activity had a much greater possibility of chance correlation between the two stains. When the percent of morphological correlation of calcium deposits with corresponding areas of alkaline phosphatase activity was plotted against the calcification gradings (Graphs 19-21), an increasing correlation was seen as the calcification grew more intense. The strongest calcification grade had the highest correlation with the alkaline phosphatase activity. This could be explained by the fact that both alkaline phosphatase activity and calcification increased in distribution and intensity with time so
that both filled the labyrinth by the end of gestation. Therefore, the areas they covered were the same. The side by side comparison contradicted the general observations made by previous authors which implied that calcium and alkaline phosphatase were found in the same location in the placenta. Although both were found lining the maternal sinuses, and towards the end of gestation they lined the same maternal sinuses, they did not always occupy the same sites in the placenta.

The original links between calcium and alkaline phosphatase were those of guilt by association. The present study showed that this association does not always exist. However, the recent isolation of calcium activated ATPases (Shami and Radde 1971 and Miller and Berndt 1973) presented evidence for the transport function of alkaline phosphatase which the studies in morphology could not easily contradict. The differences would seem to be irreconcilable if a proposal derived from the Wislocki et al (1946) calcification model were adopted. Calcification at the sites of transport would be a necessary result of halting the removal of calcium by the fetal blood stream if (i) the alkaline phosphatase provided the phosphate ions that would combine with the calcium ions in the blood to form insoluble calcium deposits in the placenta and (ii) these deposits were absorbed by the labyrinthine cells and transferred to the fetal blood stream. However, if a transport system which did not combine the calcium with the phosphate ion could be constructed, the problem of the low morphological correlation could be explained. Jardetzky (1966) proposed a general model for active transport of ions across cell membranes which, when applied to calcium transport, would keep the calcium in the ionic form.
Jardetzky's (1966) model hypothesized that there were proteins incorporated into a cell membrane which folded into a specific three-dimensional conformation specially suited for binding the calcium ion. Once the calcium ion was bound, the enzyme would be phosphorylated by ATP which would provide the energy for a change in conformation. The change in conformation would open a channel to the inside of the cell and the calcium ion would diffuse from the pumping site. After the calcium ion left the binding site, the enzyme would return to its original configuration, closing the channel to the inside of the cell and exposing the binding site to the outside to start the process once again. The calcium ion would remain soluble throughout the transport process and could diffuse from the sites of phosphatase activity and precipitate in places where such activity was not necessarily high. Placental calcification would not be dependent on the phosphate ion concentration but on the concentration of any anion whose calcium salt has a low solubility constant. The calcification in the placenta would then be subject to local conditions and concentrations of anions. This would lead to randomized sites of calcification not associated necessarily with alkaline phosphatase activity. The ligation of the umbilical cord would still be the ultimate cause of the calcium deposition by causing the cessation of removal of calcium ions leading to the increase in concentration of calcium thus causing the formation of precipitate within the placental labyrinth.

Although the model of calcium transport presented is speculative, it is consistent with the results obtained with regard to calcification and alkaline phosphatase activity in the placenta. Any models for the
calcium pump proposed in the future must take into account the findings indicating that after cessation of fetal blood flow, i.e., the removal of transported calcium ions, calcification need not occur at the site of enzymatic activity.
SUMMARY AND CONCLUSIONS

A. Ligation of the umbilical cord in utero induced certain morphological changes in the placentae. They were:

1. Obliteration of fetal capillaries within labyrinthine plates and swelling of labyrinthine plates were observed in all experimental placentae.

2. Basophilic granular material within the labyrinthine plates and a disruption of normal arrangement of labyrinthine plates were found in the 15/48, 16/24, 16/48 and 19/24 experimental placentae.

3. Enucleation of labyrinthine cells was observed in the 19/24 experimental placentae.

B. Sites of calcification were found in both control and experimental placentae. The major findings were:

1. Calcification resulting from umbilical cord ligation was found primarily in the labyrinth of the rat placentae.

2. Calcification in the experimental placentae occurred in the connective tissue of the labyrinthine plates, lined the maternal sinuses and increased in intensity as gestation progressed.

3. The GBHA method of Kashiwa and House (1964) was more sensitive of calcium in the placentae than was the alizarin red method of Dahl (1952).
C. Alkaline phosphatase activity was found in both experimental and control placentae. The major findings were:

1. Alkaline phosphatase activity was found in the labyrinth, lining the maternal sinuses when the activity was present.

2. Alkaline phosphatase activity in the younger placentae presented a patchy distribution within the labyrinth of the placenta.

3. The patches of alkaline phosphatase activity increased in size and intensity as gestation progressed until most, if not all, of the labyrinth was covered with activity near term.

D. The comparison of the distributions of calcium deposition and alkaline phosphatase activity yielded the following findings:

1. The morphological correlation between the distribution of alkaline phosphatase activity and of calcium in the experimental placentae was low during early gestation and increased with increasing placental age and with increasing intensity of calcification.

2. It was concluded that, due to the low correlation between the distribution of alkaline phosphatase and calcium deposits during the early stages of gestation, the sites of deposition of calcium were not primarily determined by the sites of alkaline phosphatase activity.
BIBLIOGRAPHY


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### Table 2

**Alizarin Red Calcium Stain**

Degree of Calcification in the Various Placental Groups

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Table 3
Comparison of GBHA and Alizarin Red Calcium Stains
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<td>Percent Morphological Correlation vs. Dated Groupings and Calcification Groupings</td>
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### Table 6

Distribution of the 6u, 7u, and 8u Control Sections
Among the Calcification Groups of GBHA and Alizarin Red Calcium Stains and the Activity Groups of Alkaline Phosphatase Stain

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<th>Alizarin Red</th>
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</table>
Percentage of Total Number of Slides from each Dated Group compared with GBHA Calcification Grades
Graph 6
15/24E-15/24C

Graph 7
15/48E-15/48C

Graph 8
16/24E-16/24C

Graph 9
16/18E-16/18C

Graph 10
19/24E-19/24C

Percentage of the Total Number of Slides from each Dated Group compared with Activity Grades of the Alkaline Phosphatase Stain

= Experimental

= Control
Comparison of GBHA and Alizarine Red Calcium Stains

Calcification Grades vs Percentage of Experimental Placentae.
Percentage of the Total Number of Slides Showing Calcification from the Dated Groups compared with Percentage of Morphological Correlation Between Calcium Deposition and Alkaline Phosphatase Activity.
Percentage of the Total Number of Slides from the Calcification Grades compared with Percentage of Morphological Correlation Between Calcium Deposition and Alkaline Phosphatase Activity
Figure 1 illustrates the relationship of the various areas of the placental and uterine structures. The maternal circulation is illustrated in the left side of the labyrinth. The free arrows indicate the flow of maternal blood through the maternal sinuses. The fetal circulation is illustrated in the right side of the labyrinth. The descending loops represent the fetal capillaries coursing within the labyrinthine plates.
A high power view of the labyrinthine structures. (730x) (17 days)

<table>
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<th>Abbreviation</th>
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<td>FC</td>
<td>Fetal Capillary</td>
</tr>
<tr>
<td>FCEC</td>
<td>Fetal Capillary Endothelial Cell</td>
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<td>LT</td>
<td>Labyrinthine Trophoblast</td>
</tr>
<tr>
<td>MS</td>
<td>Maternal Sinus</td>
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The labyrinthine trophoblast forms the walls of the maternal sinuses. Note the fetal capillary contained within the labyrinthine trophoblast. The fetal red blood cells are nucleated whereas the maternal blood cells are enucleated, smaller, and have a typical biconcave disc conformation.
Figure 3a

A view of the labyrinth of a 15day/24hour control placenta (244x)

Figure 3b

A view of the labyrinth of a 15day/24hour experimental placenta (244x)

LP = Labyrinthine Plate
MS = Maternal Sinus

Note the swollen labyrinthine plates in 3b as compared with the labyrinthine plates in 3a.
Figure 4a
A view of the labyrinth of a 15day/48hour control placenta (244x)

Figure 4b
A view of the labyrinth of a 15day/48hour experimental placenta (244x)

BMW = Basophilic Granular Material
EMS = Enlarged Maternal Sinus
LP = Labyrinthine Plate
MS = Maternal Sinus

The enlarged maternal sinuses and the deposits of basophilic granular material within the labyrinthine plates as shown in Figure 4 are characteristic of the changes which occur after umbilical cord ligation. (Compare with the control placenta in Figure 4a).
Figure 5a
A view of the labyrinth of a 16day/24hour control placenta (244x)

Figure 5b
A view of the labyrinth of a 16day/24hour experimental placenta (244x)

EMS = Enlarged Maternal Sinus
LP = Labyrinthine Plate
MS = Maternal Sinus

The swollen labyrinthine plates and the enlarged maternal sinuses illustrated in Figure 5b are characteristic of the pathological changes occurring after umbilical cord ligation. (Compare with the control placenta illustrated in Figure 5a). The basophilic granular material does not show well due to the poor fixation of the 16/24 placentae.
Figure 6a
A view of the labyrinth of a 16day/48hour control placenta (244x)

Figure 6b
A view of the labyrinth of a 16day/48hour experimental placenta (244x)

BGM = Basophilic Granular Material
EMS = Enlarged Maternal Sinuses
LP = Labyrinthine Plates
MS = Maternal Sinus

The enlarged maternal sinuses and the deposits of basophilic granular material within the labyrinthine plates illustrated in Figure 6b are characteristic of the pathological changes occurring after umbilical cord ligation. (Compare with the control placenta illustrated in Figure 6a). Note the relatively large number of maternal sinuses cut in a longitudinal direction in the control placenta (Figure 6a) and the large number of maternal sinuses cut in cross section in the experimental placenta.
The enlarged maternal sinuses and the deposits of basophilic granular material within the labyrinthine plates illustrated in Figure 7b are typical of the pathological changes occurring after umbilical cord ligation. Note the lack of labyrinthine cell nuclei which is also characteristic of the changes resulting from umbilical cord ligation of the 19 day conceptus.
Figure 8
An example of GBHA Grade 1 and 2 calcification (no labyrinthine calcification 244x) (15/24E)

Figure 9
An example of GBHA Grade 3 calcification (weak labyrinthine calcification 244x) (16/48E)

Ca = Calcium Deposits
LP = Labyrinthine Plates
MS = Maternal Sinus

Figure 8. Note the absence of calcium stain within the labyrinth leaving only the background of counterstain.

Figures 9, 10 and 11. These three figures illustrate the increasing amount of calcium deposited in grades 3, 4 and 5 of GBHA calcification. Note that the calcium deposits form around the maternal sinuses.
Figure 10
An example of GBHA Grade 4 calcification (moderate labyrinthine calcification 244x) (16/48E)

Figure 11
An example of GBHA Grade 5 calcification (strong labyrinthine calcification 244x) (19/24E)

Ca = Calcium Deposits
LP = Labyrinthine Plates
MS = Maternal Sinus
Figure 12
An example of Alizarin Red Grade 1 calcification (no labyrinthine calcification 244x) (16/48E)

Figure 13
An example of Alizarin Red Grade 2 calcification (weak labyrinthine calcification 244x) (16/24E)

Ca = Calcium Deposits
LP = Labyrinthine Plate
MS = Maternal Sinus

Figure 12. Note the absence of calcium stain leaving only the light residual staining of the alizarin red.

Figures 13, 14, 15 and 16. This series of figures shows the increasing intensity of calcium staining in grades 2, 3, 4 and 5 respectively of alizarin red stain. Note the localization of the calcium within the labyrinthine plates near the maternal sinuses.
Figure 14
An example of Alizarin Red Grade 3 calcification (moderate labyrinthine calcification 244x) (19/24E)

Figure 15
An example of Alizarin Red Grade 4 calcification (moderately strong labyrinthine calcification 244x) (19/24E)

Ca = Calcium Deposits
LP = Labyrinthine Plates
MS = Maternal Sinus
Figure 16
An example of Alizarin Red Grade 5 calcification (strong labyrinthine calcification 244x) (19/24E)

Figure 17
An example of Grade 1 Alkaline Phosphatase activity as measured by the Burstone (1958) technique (weak labyrinthine alkaline phosphatase activity 244x) (15/48C)

APA = Alkaline Phosphatase Activity
Ca = Calcium Deposit
LP = Labyrinthine Plate
MS = Maternal Sinus

Figure 17. The localization of the alkaline phosphatase activity around the maternal sinus is illustrated. Note that the activity does not line all maternal sinuses producing a patchy effect.
Figure 18

An example of Grade 2 Alkaline Phosphatase activity as measured by the Burstone (1958) technique (moderately weak labyrinthine alkaline phosphatase activity 244x) (15/24E)

Figure 19

An example of Grade 3 Alkaline Phosphatase activity as measured by the Burstone (1958) technique (moderate labyrinthine alkaline phosphatase activity 244x) (15/48E)

APA = Alkaline Phosphatase Activity
LP = Labyrinthine Plate
MS = Maternal Sinus

Figures 18, 19, 20 and 21. This series of figures shows the increasing intensity of the alkaline phosphatase staining reaction. Note the increasing intensity of the stain and the increasing distribution in the labyrinth. In Figure 21, the activity spreads to the connective tissue of the labyrinth.
Figure 20
An example of Grade 4 Alkaline Phosphatase activity as measured by the Burstone (1958) technique, (moderately strong labyrinthine alkaline phosphatase activity 244x) (19/24E)

Figure 21
An example of Grade 5 Alkaline Phosphatase activity as measured by the Burstone (1958) technique (strong labyrinthine alkaline phosphatase activity 244x) (19/24C)

APA = Alkaline Phosphatase Activity
LP = Labyrinthine Plate
MS = Maternal Sinus
Approval Sheet

The thesis submitted by Eric R. Sigmond has been read and approved by three members of the faculty of the Graduate School.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

Jan 8, 1976
Date

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Signature of Adviser